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EXPRESSION, PURIFICATION AND CHARACTERIZATION OF EXTRADIOL DIOXYGENASE CarBb INVOLVED IN CARBAZOLE DEGRADATION PATHWAY

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Abstract

Extradiol dioxygenase is a family of enzymes essential for ring cleavage reactions in aromatic compounds degradation pathways. Extradiol dioxygenases belonging to the majority of aromatic compound degradation pathways are single peptide proteins, while a small subset of in this family was reported to be two subunits complex proteins. The extradiol dioxygenase CarB protein is a protein complex consisting of catalytic subunit CarBb with a smaller subunit CarBa. This enzyme was reported to show no ring cleavage activity without the expression of both peptides. However, to date, there was no specific study to confirm CarBb protein dependency on CarBa protein for its ring cleavage activity. In this study, we cloned, heterologously expressed and purified CarBb in *E. coli*. CarBb protein showed appreciable ring cleavage activity without expression of CarBa protein. The K_m and V_{max} values calculated were 163.68 µM and 1.19 µM/min. The effects of pH and temperature suggested that the CarBb protein was significantly unstable, suggesting that the CarBa protein may be responsible for the structural stability of the CarBb protein to function as an effective ring cleavage enzyme.

INTRODUCTION

Hydrocarbon contaminations due to anthropogenic activities are well-acknowledged and bioremediation is our best solution yet to overcome this universal environmental problem. As microorganisms with contaminant compounds degrading abilities have been isolated even in the Antarctic region, efforts to study these microbes are continuously advancing in order to develop improved bioremediation solutions [1-3]. Carbazole, a compound produced from impurities of crude oil, shares a similar planar structure of dioxin and dibenzofuran molecules (Figure 1). Dioxin and dibenzofuran molecules are regarded as recalcitrant environmental pollutants with carcinogenic properties [4]. Isolation of fungal and bacterial strains capable of utilizing carbazole as carbon, nitrogen and energy source from soil, freshwater and seawater environments have been reported [5,6].

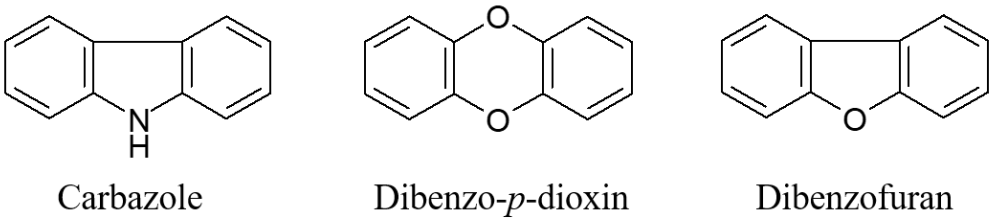

Figure 1. Chemical structures of carbazole, dibenzo-*p*-dioxin and dibenzofuran

For effective bioremediation application development, the biochemical reactions and genes responsible for the degradation of heterocyclic compounds have been extensively studied [7,8]. Similar to most polycyclic aromatic hydrocarbon degradation, in the carbazole degradation pathway, 2 oxygen atoms were added to carbazole through angular dioxygenation [9,10] to produce a diol derivative which is later converted to 2'-aminobiphenyl-2,3-diol through spontaneous ring cleavage. Extradiol dioxygenase then catalyzes the ring cleavage at the C-C bond adjacent to the vicinal hydroxy groups in a process called *meta*-cleavage (Figure 2). In contrast, *ortho*-cleavage is the process of cleaving carbon bonds between vicinal hydroxy groups. Product of *meta*-cleavage activity will be hydrolyzed by a *meta*-cleavage product hydrolase, which will transform substrates for downstream pathway before assimilation through TCA cycle [11,12].

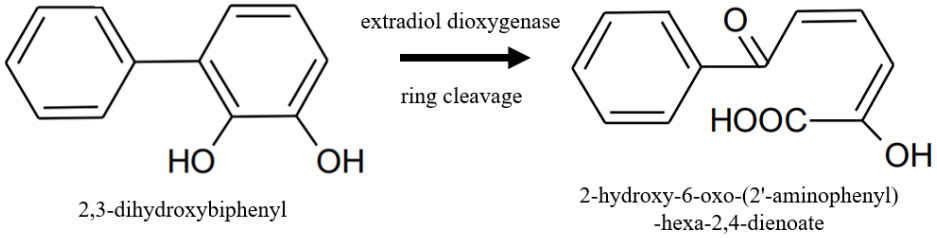

Figure 2. Estradiol dioxygenase (CarBb) ring cleavage (*meta*-cleavage) activity towards biphenyl-type substrate.

Among the earliest *car* genes isolated and studied were from *Pseudomonas resinovorans* strain CA10 and *Sphingomonas* sp. strain KA1 [13,14]. These studies have facilitated further isolations of novel gene clusters involved in carbazole degradation from different environments [15,16]. Extradiol dioxygenase of strain CA10 is a class III extradiol dioxygenase designated CarB are coded by *carBb* and *carBa* genes. Although most extradiol dioxygenases are homo-multimeric, CarB is made of two proteins, CarBb, a larger subunit that carries the catalytic site and CarBa, a smaller subunit which function remains unknown. Although the catalytic site of this enzyme is on the larger subunit, it was reported that activity was only detected when both proteins were expressed. CarBaBb showed strong ring cleavage activity towards biphenyl-type substrates but showed weak activity towards catechol-type substrates [17,18].

A number of crystal structures of ring cleavage extradiol dioxygenase have been studied. However, extradiol dioxygenase LigAB is the only class III member studied through its crystal structures. The overall structure of LigAB showed that it is different from class II enzymes. The 2 histidine residues and a single glutamic acid which coordinates Fe ion were all located in the large subunit LigB where the catalytic reaction occurs. These 3 residues are also well conserved by other members of class III. However, the geometrical coordination of elements in the active site was similar to class II enzymes, implying same reaction mechanism for all classes of extradiol dioxygenase [20,21]. Studies on other extradiol dioxygenases such as BphC and LigAB revealed many details on the reaction mechanism of these ring-cleaving enzymes [22]. However, the significance of the small subunit in hetero-multimeric extradiol dioxygenase remains unknown. In this study, we investigated the role of CarBa subunit in CarBaBb hetero-tetramic extradiol dioxygenase. The role of CarBa was deduced by a comparison of CarBaBb characteristics with active CarBb protein.

MATERIALS AND METHODS

Bacterial Strains, Vector and Medium

Escherichia coli strains XL1-Blue and BL21(DE3) (Nippon Gene, Tokyo, Japan) were used for plasmid construction and protein expression. Expression plasmids were constructed based on pET-15b. *E. coli* strains were grown on LB medium containing appropriate antibiotics (final concentration of 100 μg/ml for ampicillin or 50 μg/ml for

kanamycin) at 37˚C with shaking (300 strokes/min for glass tubes or 120 rpm for baffled flasks). When cultures were maintained in plates, LB medium containing 1.5% (w/v) agar powder were used. Protein expression in BL21(DE3) strain was induced using isopropyl-β-D-thiogalactopyranoside (IPTG).

Expression Vector Construction

The *car*Bb gene of strain CA10 which originally reported by Sato et al., 1997, was synthesized through gene synthesis service (GENEWIZ, Suzhou, China) based on the sequence deposited in GenBank (accession no. AB047548.1). Forward primer nucleotides included an *Xba*I site and ribosome binding site sequences upstream of the initial codon in the amplified products. Reverse primer nucleotides incorporated an *Xho*I site into the PCR products. In order to facilitate protein purification, codons for coding 6×His were also added upstream of end codon of the gene. The amplified product was purified and ligated into a T-vector pT7Blue prior to subcloning between the *Xba*I and *Xho*I site of pET-15b vector. The constructed expression vector was designated p15-Bb for the expression of CarBb. The constructed expression vector was sequenced for errors. For coexpression of CarBb with CarBa protein, protein expression procedures were conducted using materials and methods described Iwata et al. [17].

Protein Expression and Purification

Strain BL21(DE3) harboring p15-Bb was inoculated into baffled flasks of 200 ml LB medium containing ampicillin, was allowed to grow at 37 °C to at least OD_{600} 1.0 before protein expression was induced by addition of IPTG (final concentration of 1 mM). After this addition, the culture continued at a lowered temperature of 25 °C for approximately 4 hours before harvest. Cells were disrupted via sonication using stainless steel microtip (Branson 450 Digital Sonifier, 0.5 sec pulses of 30 secs at 30% power output) with sample tube submerged in ice. Crude protein extract was centrifuged to separate soluble and insoluble fractions. Soluble fractions were used for the purification step. Purification procedures were conducted at 4˚C utilizing Akta FPLC system (GE Healthcare Life Sciences, USA). The purification was conducted in a 2-step fashion, metal chelation affinity and gel filtration chromatography. Purified protein samples were visualized using SDS-PAGE (15% gel concentration) and protein concentrations were determined using the method of Bradford.

Ring Cleavage Activity Assay

For measuring ring cleavage activity at wide range of pH, buffers used were citrate buffer (pH 3.0 – 6.0), 2-(N-morpholino) ethanesulfonic acid (MES)-NaOH buffer (pH 5.5 – 7.0), 3-(N-morpholino) propanesulfonic acid (MOPS)-NaOH buffer (pH 6.5 – 8.0), Tris-HCl buffer (pH 7.5 – 9.0) and glycine-NaOH buffer (pH 8.5 – 10.5). Buffer strength for each buffer solution was set to 50mM and temperature 25˚C was maintained for all ring cleavage activity assay. To measure ring cleavage activity for variable temperatures, measurements were performed at pH 7.5 and temperatures of 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55 and 60˚C. Enzyme thermal stability was evaluated by measuring remaining *meta*-cleavage activity after 30 min of incubation at 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55 and 60˚C. For all ring cleavage activity assay, substrate used was 2,3-dihydroxybiphenyl (2,3-DHBP) (final concentration 100 μM).

Substrate Specificity Analysis

In order to determine substrate specificity for CarBb, appropriate amount of purified CarBb enzyme was used to cleave 2,3-dihydroxybiphenyl at different concentrations in a 1 ml scale reaction at 25 °C. Absorption wavelength 434 nm and extinction coefficients of ring fission products 13.2 mM^{-1} cm^{-1} were used for analysis.

RESULTS AND DISCUSSION

Expression and Purification

CarBb was expressed under the influence of *T7lac* promoter from pET vector system. Strain BL21(DE3) harboring vector p15-Bb showed no band on SDS-PAGE gel corresponding CarBb protein at growth temperatures 30 ˚C and 37 ˚C. Further investigation revealed that at these temperatures, expressed CarBb collected mostly in the insoluble fraction of the crude protein extract (data not shown). To obtain more proteins insoluble fraction, growth conditions were altered to express CarBb at a lower temperature after IPTG induction. Active CarBb protein was recovered when the expression was induced at 25˚C. The initial step of purification was performed using affinity chromatography. CarBb protein eluted as a single dominant peak. Similar results were also obtained for the 2nd step of purification using gel filtration. Summary of purification is shown in Table 1 and purified CarBb protein was confirmed by SDS-PAGE (Figure 3).

Table 1. Summary of purification steps for CarBb protein expressed in *E. coli* BL21(DE3).

Purification step	Volume (ml)	Total protein (mg)	Total activity (U)	Specific activity (10^{-2} U/mg)	Purification fold
Cell extract	60	1402	136	9.7	-
Affinity chromatography	9	53	21	39.7	4.1
Gel filtration	9	44	28	63.6	6.5

One unit (1 U) of activity was defined as the amount of enzyme needed to form 1 µmol of *meta*-cleavage compound per minute at 25 °C.

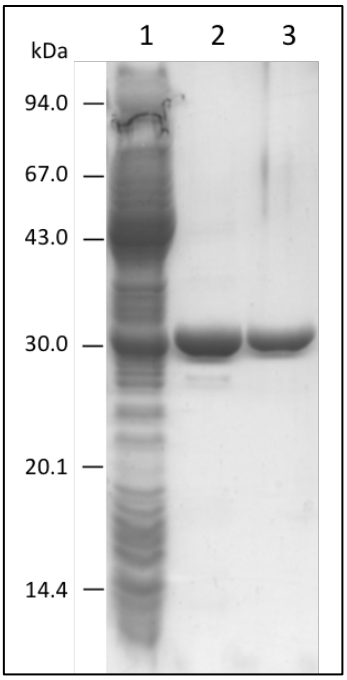

Figure 3. SDS-PAGE showing each step of purification. Lanes (1) crude protein extract, CarBb protein after (2) affinity chromatography and after (3) gel filtration chromatography. CarBb protein was purified to homogeneity after 2 steps chromatography.

Although the study of CarBaBb of strain CA10 was already reported [17], in this study, the characterization of only CarBb subunit provided insights into the function of small subunit carBa, which is still unknown. This study disapproves the previous report that the expression of both catalytic subunit CarBb and small subunit CarBa is indispensable for the carbazole degradation pathway's ring cleavage activity. However, the activity was significantly reduced to a hundred order without the small subunit coexpressed. This low activity was not clear as the active site containing Fe ion coordinated by well-conserved residues is located in CarBb subunit. The CarBa subunit may play a role in providing structural stability, as suggested in the case for LigAB ring cleavage protein complex involved in lignin degradation [20].

Substrate Specificity

The Michaelis-Menten constant K_m and maximum reaction velocity V_{max} values for CarBb were deduced to determine the substrate specificity of this enzyme towards 2,3-DHBP. The values were deduced using a Lineweaver-Burk plot. The K_m and V_{max} values calculated were 163.68 µM and 1.19 µM/min.

According to the reported results of Iwata et al. [17], the K_m value for CarBb coexpressed with carBa protein toward substrate 2,3-dihydroxybiphenyl was 1.9 µM. When compared to the K_m value of CarBb protein in this study, it can be concluded that the ability for CarBb protein to bind to substrate was significantly affected without coexpression of CarBa protein. Despite conserved amino acid residues necessary for catalysis of ring cleavage are available exclusively in CarBb peptide, the interaction with CarBa protein may contribute to the formation of optimized 3D structure, specifically needed for enzyme-substrate complex formation. The substrate specificity K_m of CarBb protein was approximately 86 fold higher than when expressed with CarBa protein. It can also be hypothesized that the small subunit peptide may be a deciding factor for the substrate selection for CarBb ring cleavage activity and modification towards the small subunit peptide may potentially allow the opportunity for engineering this ring cleavage enzyme.

Effects of Temperature and pH on Activity of CarBb

Effects of temperature and pH on the activity of CarBb was tested over a wide range of conditions. Enzymatic activity was highest when reaction occurred at the temperature of 15 °C (Figure 4), which is a relatively low temperature. The activity was below 50% after the temperature 25 °C. For pH, the *meta*-cleavage activity was highest at pH 7.5 and the activity was below 50% when reaction occurred outside of pH 7-8 range (Figure 5).

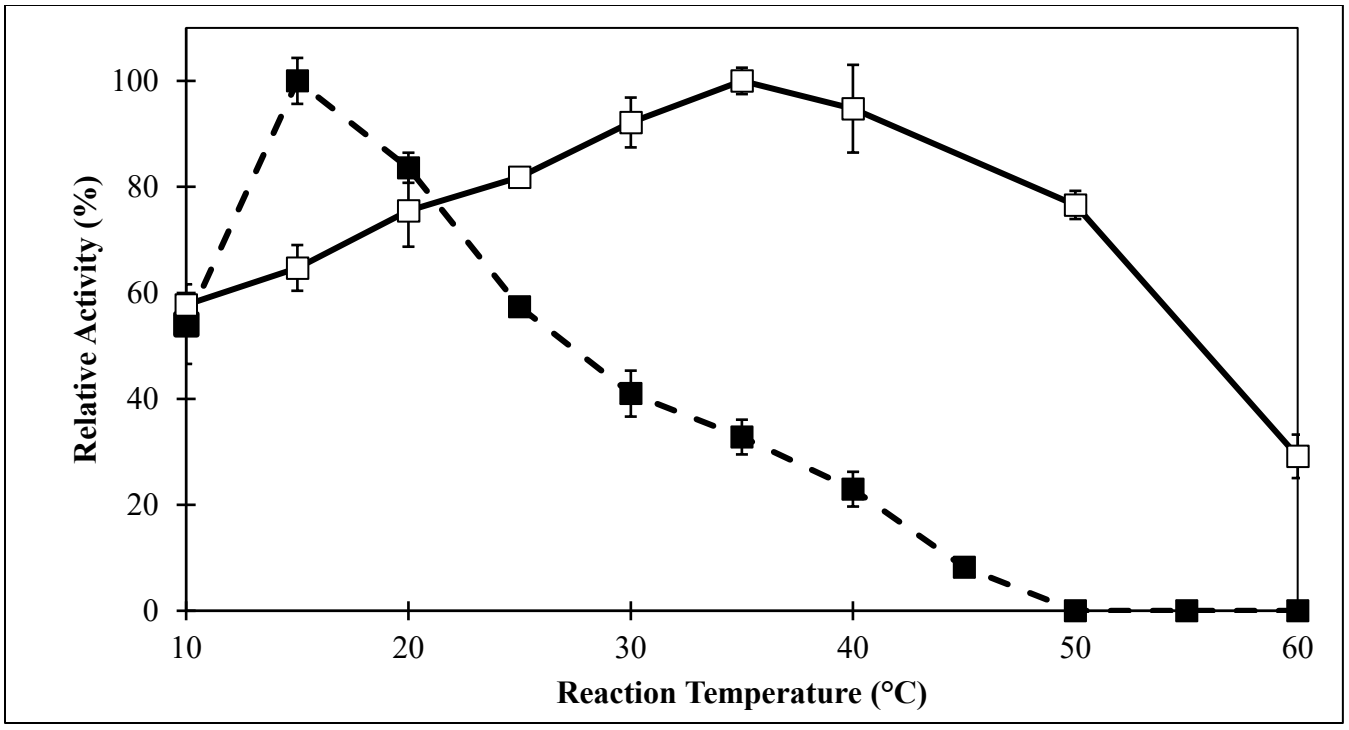

Figure 4. Enzymatic activities at various temperatures. Dotted line represent the activities of CarBb and solid line repesents activities of CarBb coexpressed with CarBa protein. Activity was measured in 50 mM Tris-HCl pH 7.0 buffer using 100 µM 2,3-DHBP as substrate.

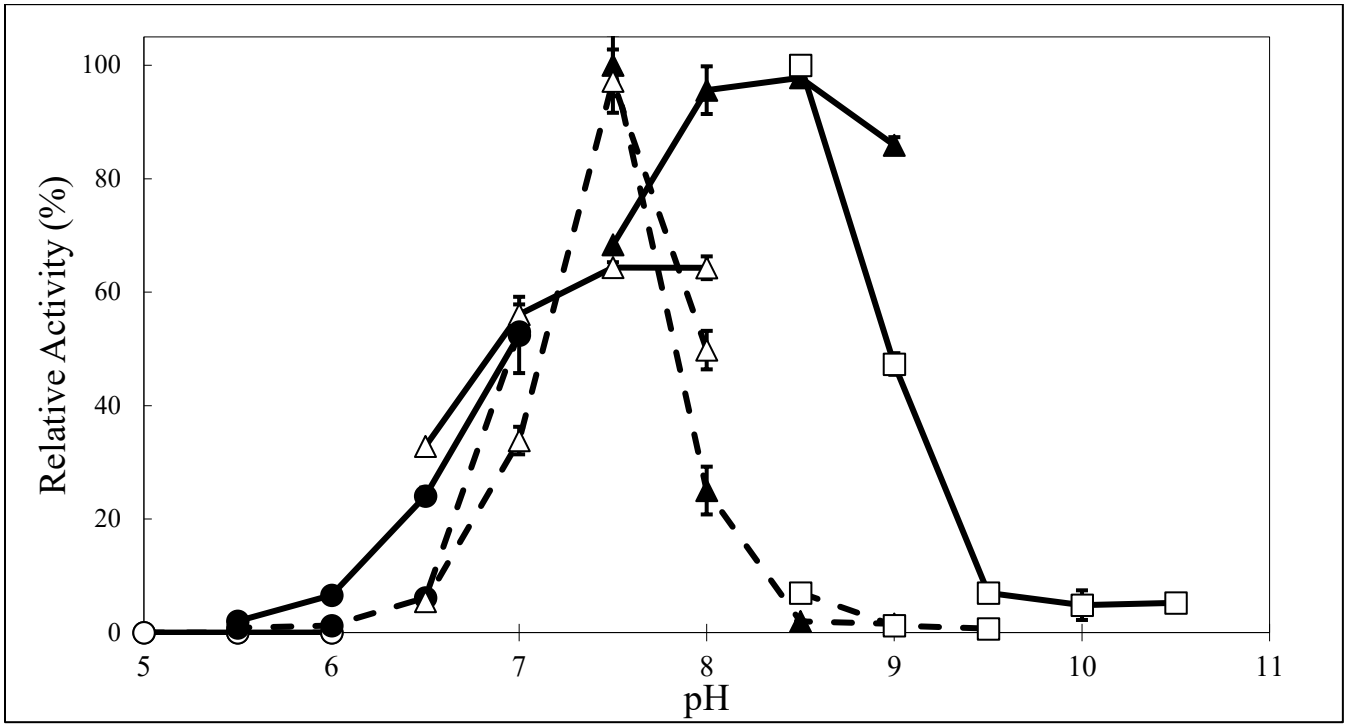

Figure 5. Enzymatic activities at various pH. Activities were measured at 25 °C buffer using 100 µM 2,3-DHBP. Buffers used were 50 mM citrate (○), 50 mM 2-(N-morpholino) ethanesulfonic acid (MES)-NaOH (●), 50 mM 3-(N-morpholino) propanesulfonic acid (MOPS)-NaOH (Δ), 50 mM Tris-HCl (▲) and 50 mM glycine-NaOH (□).Dotted line represent the activities of CarBb and solid line repesents activities of CarBb coexpressed with CarBa protein.

The characteristics of CarBb were almost similar when compared to CarBb coexpressed with CarBa protein except for the effect of temperatures. This indicated that CarBb was more thermally unstable without CarBa protein. However, the instability of CarBb did not cause it to denature as purification was possible from soluble fractions. The similarities in many ways also dismiss the role of CarBa indirectly interacting with substrate. A similar test should also be conducted on other 2 subunit *meta*-cleavage enzymes other than CarBaBb, such as LigAB from *Sphingomonas paucimobilis* strain SYK-6 and FlnD1D2 from *Rhodococcus* sp. Strain DFA3 [23]. It would also be insightful to investigate whether CarBb can be reconstituted with CarBa *in vitro* and see how far the small subunit supports the stability of the large subunit. This study did not rule out other possible roles and/or functions of the small subunit peptide. There may also be a relationship between the small subunit peptide and the enzyme inactivation due to ferrous oxidation as the redox state of non-heme Fe ion located in the active site vicinity is crucial for ring cleavage reaction.

Even though the structural study of LigAB revealed many details of a hetero-multimeric extradiol dioxygenase, several significant differences should be considered. Despite similarities, such as the conserved Fe ion ligands in the large subunits, small subunit LigA and CarBa are very distinct. LigA is composed of 139 amino acid residues, while CarBa has only 90 amino acids with low similarities. The difference in substrate also suggests changes at the active site. For LigAB, the substrate is protocatechuate while the substrate for CarBaBb is 2'-aminobiphenyl-2,3-diol, different enough to warrant changes in each active site. Being a 2 subunit class III extradiol dioxygenase with a biphenyl-type substrate, this places CarBaBb in between LigAB and BphC characteristics. A 3D structural study of CarBaBb may reveal novel features among extradiol dioxygenases.

CONCLUSION

In this study, we report successful expression, purification and characterization of extradiol dioxygenase CarBb involved in carbazole degradation pathway with active ring cleavage activity without the coexpression of small subunit protein, CarBa. The expressed CarBb protein showed significantly higher K_m values and low activity at higher temperatures. This study concluded that small subunit portein CarBa is crucial for maintaining structural stability CarBb but did not directly involve in ring cleavage catalytic activity.

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REFERENCES

1. Lee, G. L. Y., Ahmad, S. A., Yasid, N. A., Zulkharnain, A., Convey, P., Wan Johari, W. L., Alias, S. A., Gonzalez-Rocha, G. and Shukor, M. Y. (2018). Biodegradation of phenol by cold-adapted bacteria from Antarctic soils. *Polar Biology*, 41: 553–562.

2. Abdulrasheed, M., Zakaria, N. N., Roslee, A. F. A., Shukor, M. Y., Zulkharnain, A., Napis, S., Convey, P., Alias, S. A., Gonzalez-Rocha, G. and Ahmad, S. A. (2020). Biodegradation of diesel oil by cold-adapted bacterial strains of *Arthrobacter* spp. from Antarctica. *Antarctic Science*, 1–13.

3. Roslee, A. F. A., Zakaria, N. N., Convey, P., Zulkharnain, A., Lee, G. L. Y., Gomez-Fuentes, C. and Ahmad, S.A. (2020) Statistical optimisation of growth conditions and diesel degradation by the Antarctic bacterium, *Rhodococcus* sp. Strain AQ5-07, *Extremophiles*, 24: 277–291.

4. Gabelova, A. (2020). 7H-Dibenzo[c,g]carbazole: Metabolic pathways and toxicity. *Chemico-Biological Interactions*, *323*, 109077. https://doi.org/10.1016/j.cbi.2020.109077

5. Azri, M. F. D. B., Zulkharnain, A. B., Husaini, A. A. S. A. and Ahmad, S. A. B. (2015). The degradation of carbazole and the production of ligninolytic enzyme by isolated marine fungi. *Journal of Chemical and Pharmaceutical Sciences*, 8: 330–335.

6. Ouchiyama, N., Zhang, Y., Omori, T. and Kodama, T. (1993). Biodegradation of Carbazole by Pseudomonas spp. CA06 and CA10. *Bioscience, Biotechnology, and Biochemistry*, *57*: 455–460.

7. Ahmad, A. B., Zulkharnain, A. B., & Husaini, A. A. S. B. A. (2015). Substrate specificity of angular dioxygenase from carbazole-degrading bacterium *Neptuniibacter* sp. strain CAR-SF. *Journal of Chemical and Pharmaceutical Sciences*, 8: 382–388.

8. Manas, N. H. A., Drus, M. N. A. M., Zulkharnain, A., Hui, J. L. C., Azelee, N. I. W. and Dailin, D. J. (2019). Transport phenomena of carbazole biodegradation by immobilized *Thalassosspira profundimaris* cell and mechanical properties. *AIP Conference Proceedings*, *2155*(1), 020009.

9. Groce, S. L. and Lipscomb, J. D. (2005). Aromatic ring cleavage by Homoprotocatechuate 2,3-dioxygenase: Role of His200 in the kinetics of interconversion of reaction cycle intermediates. *Biochemistry*, 44: 7175–7188.

10. Harayama, S. and Rekik, M. (1989). Bacterial aromatic ring-cleavage enzymes are classified into two different gene families. *Journal of Biological Chemistry*, 264: 15328–15333.

11. Nojiri, H., Habe, H. and Omori, T. (2001). Bacterial degradation of aromatic compounds via angular dioxygenation. *Journal of General and Applied Microbiology*, 47: 279–305.

12. Fuse, H., Takimura, O., Murakami, K., Inoue, H. and Yamaoka, Y. (2003). Degradation of chlorinated biphenyl, dibenzofuran, and dibenzo-p-dioxin by marine bacteria that degrade biphenyl, carbazole, or dibenzofuran. *Bioscience, Biotechnology, and Biochemistry*, 67: 1121–1125.

13. Inoue, K., Habe, H., Yamane, H., Omori, T. and Nojiri, H. (2005). Diversity of carbazole-degrading bacteria having the car gene cluster: Isolation of a novel gram-positive carbazole-degrading bacterium. *FEMS Microbiology Letters*, 245: 145–153.

14. Nojiri, H., Sekiguchi, H., Maeda, K., Urata, M., Nakai, S.-I., Yoshida, T., Habe, H. and Omori, T. (2001). Genetic characterization and evolutionary implications of a car gene cluster in the carbazole degrader *Pseudomonas* sp. strain CA10. *Journal of Bacteriology*, 183: 3663–3679.

15. Maeda, R., Nagashima, H., Zulkharnain, A. B., Iwata, K. and Omori, T. (2009). Isolation and characterization of a car gene cluster from the naphthalene, phenanthrene, and carbazole-degrading marine isolate *Lysobacter* sp. strain OC7. *Current Microbiology*, 59: 154–159.

16. Nagashima, H., Zulkharnain, A. B., Maeda, R., Fuse, H., Iwata, K. and Omori, T. (2010). Cloning and nucleotide sequences of carbazole degradation genes from marine bacterium *Neptuniibacter* sp. strain CAR-SF. *Current Microbiology*, 61: 50–56.

17. Iwata, K., Nojiri, H., Shimizu, K., Yoshida, T., Habe, H. and Omori, T. (2003). Expression, purification, and characterization of 2′-aminobiphenyl-2,3-diol 1,2-dioxygenase from carbazole-degrader pseudomonas resinovorans strain CA10. *Bioscience, Biotechnology and Biochemistry*, 67: 300–307.

18. Larentis, A. L., Alves, T. L. M. and Martins, O. B. (2005). Cloning and expression of meta-cleavage enzyme (CarB) of carbazole degradation pathway from *Pseudomonas stutzeri*. *Brazilian Archives of Biology and Technology*, 48: 127–134.

19. Uragami, Y., Senda, T., Sugimoto, K., Sato, N., Nagarajan, V., Masai, E., Fukuda, M. and Mitsui, Y. (2001). Crystal structures of substrate free and complex forms of reactivated BphC, an extradiol type ring-cleavage dioxygenase. *Journal of Inorganic Biochemistry*, 83: 269–279.

20. Sugimoto, K., Senda, T., Aoshima, H., Masai, E., Fukuda, M. and Mitsui, Y. (1999). Crystal structure of an aromatic ring opening dioxygenase LigAB, a protocatechuate 4,5-dioxygenase, under aerobic conditions. *Structure*, *7*: 953–965.

21. Barry, K. P. and Taylor, E. A. (2013). Characterizing the promiscuity of LigAB, a lignin catabolite degrading extradiol dioxygenase from *Sphingomonas paucimobilis* SYK-6. *Biochemistry*, 52: 6724–6736.

22. Barry, K. P., Ngu, A., Cohn, E. F., Cote, J. M., Burroughs, A. M., Gerbino, J. P. and Taylor, E. A. (2015). Exploring allosteric activation of LigAB from *Sphingobium* sp. strain SYK-6 through kinetics, mutagenesis and computational studies. *Archives of Biochemistry and Biophysics*, 567: 35–45.

23. Kotake, T., Matsuzawa, J., Suzuki-Minakuchi, C., Okada, K., Nojiri, H. and Iwata, K. (2016). Purification and partial characterization of the extradiol dioxygenase, 2′-carboxy-2,3-dihydroxybiphenyl 1,2-dioxygenase, in the fluorene degradation pathway from *Rhodococcus* sp. strain DFA3. *Bioscience, Biotechnology and Biochemistry*, 80: 719–725.